

## Effects of Ylang-Ylang Essential Oil on the Relaxation of Rat Bladder Muscle in vitro and White Rabbit Bladder in vivo

Current and primary treatment modality in overactive bladder includes the administration of anticholinergics. The demand for new agents has been rising since anticholinergics have proven to come with many side effects. This study was designed to investigate the effects of ylang-ylang essential oil (YYEO) on the relaxation of urinary bladder muscle in vitro and in vivo. Effects of YYEO were assessed on resting tension, and electrical field stimulation- and various drug-induced contraction in vitro by checking the isometric tension changes of muscle strips and same procedures were repeated in the presence of methylene blue, Nw-Nitro-L-arginine methyl ester hydrochloride (L-NAME), or N-ethylmaleimide, and in vivo. YYEO decreased significantly the contractility of strips. There was no statistically significant difference between the treated group only with YYEO and the pre-treated group with YYEO and methylene blue or L-NAME. When N-ethylmaleimide was employed, there was a statistically significant decrease in the rate of contraction. In vivo studies showed the same results compared with in vitro study. The results of this study indicate that YYEO has a relaxing effect on the bladder, and such mechanism is thought to be brought about by a pathway mediated by c-AMP.

**Key Words :** Oils, Volatile; Neurogenic Bladder; Muscle Hypertonia; Cholinergic Antagonists

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Received : 10 September 2002  
Accepted : 23 January 2003

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\*The research was conducted by the research  
fund of Dankook University in 2001.

## INTRODUCTION

In overactive bladder, especially neurogenic detrusor overactivity that arises in patients with spine disorder above sacral micturition center, there are many bladder irritative symptoms due to low bladder compliance, neurogenic detrusor overactivity and high intravesical pressure (1-3). In those cases, there are many complications including recurrent urinary tract infection and vesicoureteral reflex causing upper urinary tract injury, eventually a renal failure. Thus the purposes of the management in the overactive bladder is to decrease the intravesical pressure to appropriate levels and to protect the upper urinary tract. The current and primary treatment modalities include the administration of anticholinergics alone or with the intermittent catheterization (4, 5). However, despite such methods are effective, the administration of anticholinergics may often be ceased due to the side effects of anticholinergics such as flushing, drowsiness, constipation, erectile dysfunction, dry mouth or in high dose, central nervous system symptoms and cardiovascular symptoms. Also the administration of anticholinergics are not recommended in cases of hyperthyroidism, congestive heart failure, liver disease, kidney disease, myasthenia gravis, and megacolon or closed angled glaucoma. Therefore the

demand for new agents with less adverse effects has been rising accordingly (6).

Aromatherapy is often used to manage the variable symptoms by abnormal regulation of autonomic nervous system such as anxiety, insomnia or immune system. Aromatherapy may be used by smelling, inhalation, footbath, or massage, only with a few side effects such as hypertension or diarrhea. Ylang-Ylang essential oil (YYEO) (*Cananga odorata*) is used worldwide as a perfume. It can also be used as intestinal antispasmodics (7, 8). Recent studies indicate the some components in YY oil have vasodilator properties and relaxing effects on smooth muscle (9-11).

This study was designed to investigate the effects of YYEO, which is currently used in aromatherapy on the relaxation of urinary bladder muscle in vitro and in vivo.

## MATERIALS AND METHODS

### Preparation of tissue strips for in vitro study

Male Sprague-Dawley rats weighing 250 to 300 g were used and anesthetized by intraperitoneal sodium pentobarbital (5 mg/kg) and exsanguinated via cervical dislocation. Following

a suprapubic midline incision, bladder was isolated and longitudinal detrusor muscle strips measuring approximately  $1 \times 10$  mm were obtained within the Krebs' solution with room temperature airing 95% O<sub>2</sub> and 5% CO<sub>2</sub> after bladder body was dissected. Each strip was mounted in organ-bath containing 20 mL Krebs' solution with the following composition (mmol/L): NaCl, 118.1; NaHCO<sub>3</sub>, 25.0; KCl, 4.6; KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.2; glucose, 11.0, and equilibrated at 37 °C with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for resulting pH 7.4. After the 90-min equilibration with frequent washout using fresh Krebs' solution, strips of detrusor muscle were loaded with resting tension of 1 g, and optimal isometric tension was obtained as evidenced by two consecutive contraction to bethanechol ( $5 \times 10^{-4}$  M) that differed by less than 10%.

Changes in isometric tension were measured by Grass FT03 transducer (Grass Instruments, Quincy, MA, U.S.A.) and recorded by Grass 79E Polygraph (Grass Instruments).

### Experimental procedure for in vitro study

The effects of YYEO on basal tension are reported as the tension at the end of the 10-min incubation in the presence of 0.05 mL YYEO. Electrical field stimulation (EFS) was delivered via platinum electrodes set on either side of the muscle strip in each organ bath. Intramural nerves were stimulated with a Grass 88 field stimulator (Grass Instruments) delivering biphasic square wave pulse of 50 V, 1.0 ms wide at 1 to 16 Hz with a 2-min interval between the stimulation. After the 30-min incubation with fresh Krebs' solution, 0.05 mL YYEO was added to each organ bath. The tissue was incubated for 10 min and then EFS was repeated.

In separate experiments, the effects of YYEO on bethanechol, ATP and KCl-stimulated contractions were investigated. After the 60-min incubation at 1 g tension, bethanechol (0.01 mM) was added to each organ bath. Peak response was recorded, and the bath was washed out to be refilled with Krebs' solution and equilibrated for 30 min. Subsequently, after the 10-min incubation in 0.05 mL YYEO, the stimulation with bethanechol (0.01 mM) was repeated. The effects were also recorded. The effects of YYEO on stimulation by ATP (2 mM) and KCl (127 mM) were assessed in the same way with other strips.

In order to investigate which drugs were effective in blocking the YYEO-induced relaxation, other separate experiments were performed. First, after the 60-min equilibration at 1 g tension, the effects of YYEO on electrical field stimulation (EFS)-, bethanechol-, ATP- and KCl-stimulated contractions were investigated with the above method. Baths were then washed out to be refilled with Krebs' solution and equilibrated for 30 min. Subsequently, after the 10-min incubation in 0.001 mM methylene blue (guanylate cyclase inhibitor), the stimulations with EFS, bethanechol, ATP and KCl were repeated. The effects were also recorded. The effects of L-NAME (Nw-Nitro-L-arginine methyl ester hydrochloride, nitric

oxide synthase inhibitor, 0.001 mM) and N-ethylmaleimide (c-AMP nonspecific inhibitor, 0.001mM) on relaxation by YY oil were assessed in the same way in other strips to evaluate which mechanism was related to relaxation by YYEO.

### Preparation of animal for in vivo study

Male New Zealand white rabbits weighing about 2.5 kg were used for this investigation. After the sedation with an intramuscular injection of ketamine (10 mg/kg), the rabbits were anesthetized and maintained with enflurane (2.5–3.5%). The animals breathed spontaneously. The rabbits were then placed supine position, and the body temperature was maintained at 37 °C using a heating pad and lamp. The polyethylene catheter (Clay Adams PE-50, NJ, U.S.A.) was introduced to bladder through the urethra. The intravesical pressure was measured by pressure transducer (Grass Instruments, Quincy, MA, U.S.A.) and recorded by Grass 79E Polygraph (Grass Instruments). The catheter was connected to a three-way stopcock, thus permitting the injection of 37 °C normal saline (10 mL/min). Intravesical pressure and vesical volume were then checked, when spontaneous bladder contraction was occurred. After half of full volume was introduced to bladder with 37 °C normal saline, changes of intravesical pressure to YY oil, bethanechol and ATP-induced stimulation were investigated. The femoral artery on one side was cannulated with polyethylene catheter (Clay Adams PE-10) and continuous monitoring of arterial pressure and heart rate were measured by pressure transducer (Grass Instruments) and recorded by Grass 79E Polygraph (Grass Instruments), when YY oil was administered through the vein of ear. The femoral catheter was connected to a three-way stopcock, thus permitting the injection of various drugs. Catheter was filled with heparinized saline (50 IU/2 hr) to prevent clotting. The femoral artery on other side was ligated to obtain the maximum effect of given concentrated drug.

### Experimental procedure for in vivo study

YYEO (0.01, 0.02, 0.05 mL) was injected intravenously, and the change of arterial pressure and heart rate were measured with 1-hr interval. The effects of YYEO on bethanechol- and ATP-stimulated bladder contractions were investigated. After a minimum of 1 hr of resting, bethanechol (0.01 mM) was injected intra-arterially and peak response of intravesical pressure was recorded. After the 60-min equilibrium, subsequently YY oil (0.0001–0.01 mL) was injected intra-arterially, and following the 1-min incubation, the stimulation with bethanechol (0.01 mM) was repeated. The effects were recorded. The effects of YYEO on stimulation by ATP (2 mM) were assessed in the same way with other rabbits.

Contractile responses were expressed as gram tension per 100 mg of tissue in in vitro study and as a percentage of the active muscle tone, compared with control in in vivo study. YYEO

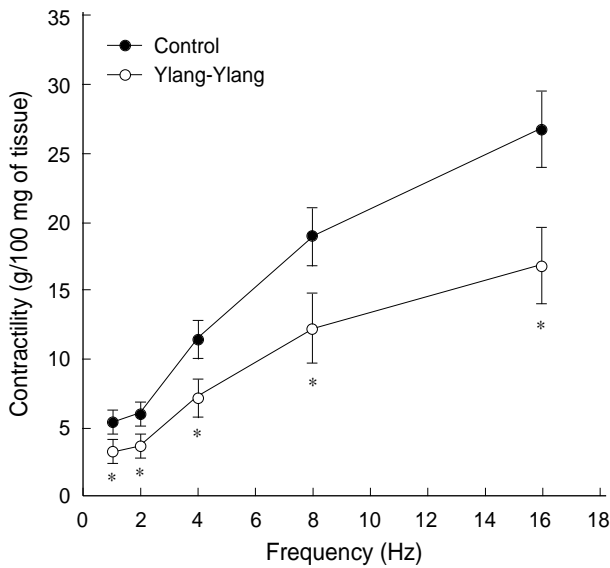


Fig. 1. Frequency-contraction curves from bladder muscle strips in male Sprague-Dawley rats. Pre-treatment with ylang-ylang oil (0.05 mL) significantly decreases the maximum tension under the control. \* $p < 0.05$  indicates significant difference from respective control,  $n = 19$  in each group.

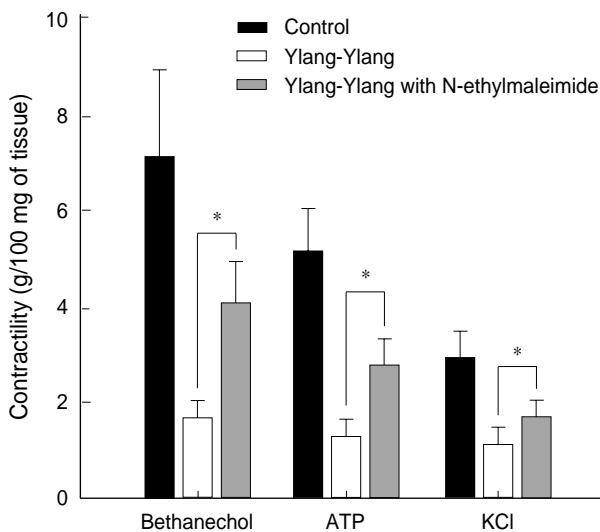


Fig. 3. Changes of the contraction induced by bethanechol, ATP and KCl after pre-treatment with ylang-ylang oil. Bethanechol, ATP and KCl-induced contractions are decreased by pre-treatment with ylang-ylang oil and N-ethylmaleimide. \* $p < 0.05$  indicates significant difference from ylang-ylang induced reduction of contractions ( $n = 8$ , bethanechol;  $n = 6$ , ATP;  $n = 6$ , KCl).

was given as a gift from the company (Aroma Newtech, Cheonan, Korea). Bethanechol chloride, ATP, KCl, L-NAME, N-ethylmaleimide and methylene blue were purchased from Sigma Co. (St Louis, MO, U.S.A.). Data were expressed as the mean (SEM), and differences were assessed using Student's  $t$ -test, with  $p < 0.05$  considered to indicate significance.

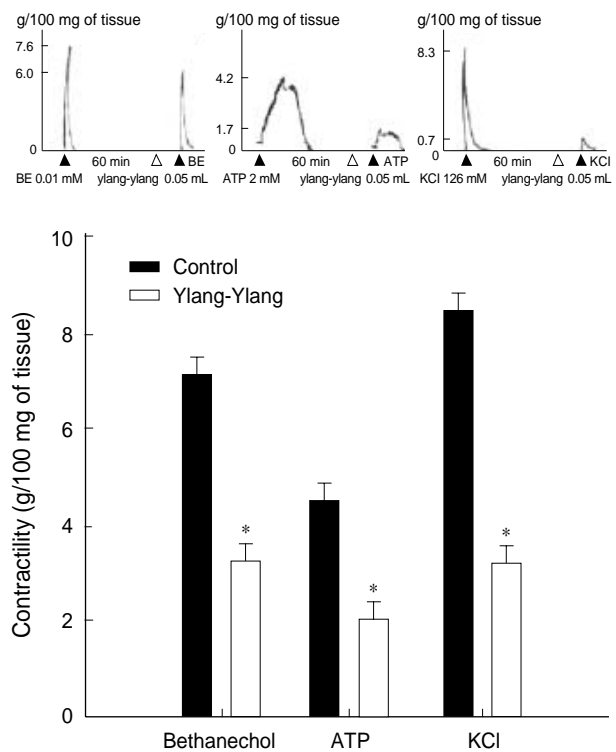


Fig. 2. Changes of the contraction induced by bethanechol, ATP, and KCl after pre-treatment with ylang-ylang oil. Bethanechol, ATP, and KCl-induced contractions are decreased by pre-treatment with ylang-ylang oil. \* $p < 0.05$  indicates significant difference from respective control ( $n = 11$ , bethanechol;  $n = 22$ , ATP;  $n = 21$ , KCl).

## RESULTS

### In vitro study

YYEO had no significant effect on the resting tone of smooth muscle strips. The response to EFS (1, 2, 4, 8 and 16 Hz) was significantly suppressed by YYEO from  $5.3 \pm 0.6$ ,  $5.8 \pm 0.7$ ,  $11.2 \pm 1.3$ ,  $18.7 \pm 2.1$ , and  $26.5 \pm 2.6$  g/100 mg to  $3.1 \pm 0.5$ ,  $3.4 \pm 0.7$ ,  $7.0 \pm 1.3$ ,  $12.0 \pm 2.5$  and  $16.0 \pm 3.1$  g/100 mg, respectively ( $p < 0.05$ ,  $n = 19$ ) (Fig. 1). Response to bethanechol, ATP and KCl were significantly suppressed by YYEO from  $7.2 \pm 0.5$ ,  $4.4 \pm 0.5$ , and  $8.6 \pm 0.8$  g/100 mg to  $3.5 \pm 0.4$ ,  $2.1 \pm 0.3$  and  $3.5 \pm 0.5$  g/100 mg, respectively ( $p < 0.01$ ,  $n = 11$ , 22, 21, respectively) (Fig. 2). YYEO-induced suppressions of all stimulation-induced contractility were not significantly changed in the presence of methylene blue or L-NAME (data not shown). All stimulation-induced contractility suppressed by YYEO was significantly restored in the presence of N-ethylmaleimide from  $1.7 \pm 0.3$ ,  $1.2 \pm 0.3$  and  $1.1 \pm 0.2$  g/100 mg (bethanechol, ATP and KCl, respectively), and  $1.0 \pm 0.2$ ,  $1.0 \pm 0.2$ ,  $2.1 \pm 0.4$ ,  $3.7 \pm 0.6$  and  $5.8 \pm 1.0$  g/100 mg (EFS) to  $4.1 \pm 0.8$ ,  $2.6 \pm 0.5$  and  $1.8 \pm 0.2$  g/100 mg, and  $1.8 \pm 0.3$ ,  $1.8 \pm 0.3$ ,  $4.1 \pm 0.8$ ,  $6.1 \pm 0.8$  and  $8.3 \pm 0.1$  g/100 mg, respectively ( $p < 0.05$ ,  $n = 10$ , 10, 11, 11,

respectively) (Fig. 3, 4).

### In vivo study

Intravesical pressure was  $16.6 \pm 2.5$  mmHg, when spontaneous bladder contraction was occurred.

During the injection of YYEO (0.01, 0.02 and 0.05 mL) intravenously, the heart rate was not significantly changed, but mean arterial pressure was decreased transiently (decrease; 0, 15 and 27.5 mmHg, respectively) (Fig. 5). Intravesical pressures induced by bethanechol and ATP in the presence of YY oil (0.0001-0.01 mL) were significantly suppressed by the amount of the above 0.001 mL YY oil from basal to  $110.1 \pm 11.4$ ,  $55.9 \pm 3.8$  and  $32.7 \pm 3.1\%$ , ( $p < 0.01$ ,  $n = 9$ ) and  $90.3 \pm 7.8$ ,  $37.7 \pm 5.8$  and  $36.1 \pm 9.3\%$ , respectively ( $p < 0.01$ ,  $n = 7$ ) (Fig. 6).

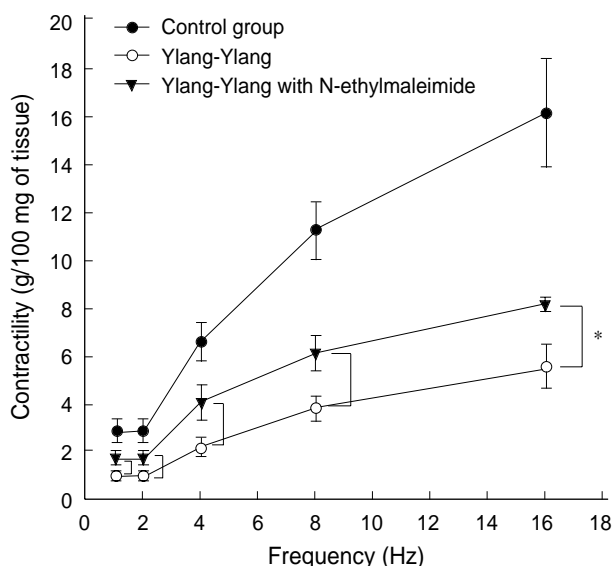


Fig. 4. Changes of the contraction induced by electrical field stimulation (EFS) after pretreatment with ylang-ylang oil. Bethanechol, EFS-induced contractions are decreased by pre-treatment with ylang-ylang oil. Reduction of contractions are not attenuated by pre-treatment with ylang-ylang oil and N-ethylmaleimide. \* $p < 0.05$  indicates significant difference from ylang-ylang induced reduction of contractions,  $n = 11$  in each group.

### DISCUSSION

YYEO is internationally used as prime component in high quality perfumes. Forty-nine different components have been identified by gas chromatography-mass spectrometry (GC-MS). Major components to list are linalool, linalyl acetate, eugenol, methyleugenol and methyl benzonate (9-11). In Brazil, methyleugenol is used in folk medicine as a stomachic and intestinal antispasmodic; it suggests YYEO makes smooth muscle of gastrointestinal system relax (7). The main component of YYEO, linalool, is used as an antibiotics as well as an antispasmodics used to treat epilepsy. Eugenol has also been widely used for many years as a topical, temporary treatment for the pain of pulpitis (12-14). Effects of YYEO on skeletal muscle and smooth muscle lead us to study the effect

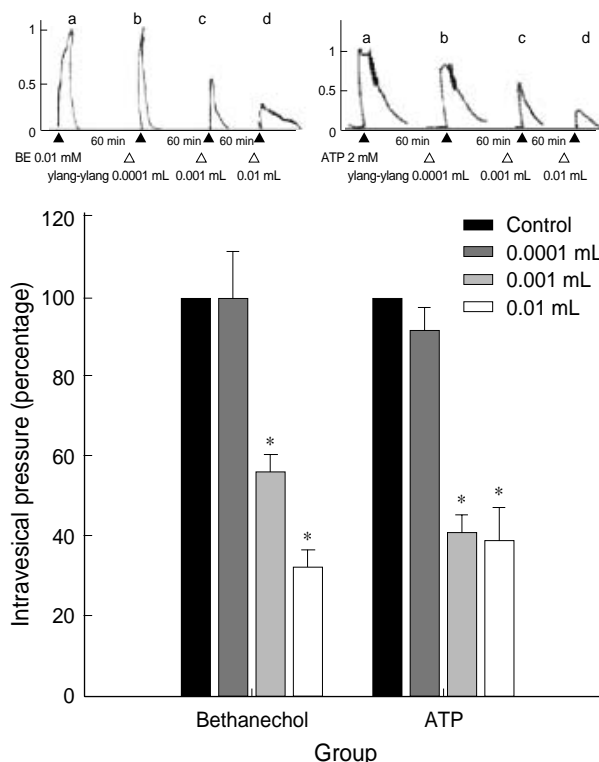


Fig. 6. Effects of pre-treated ylang-ylang oil on the response of bethanechol, and ATP. \* $p < 0.01$ , significant difference from respective control ( $n = 9$ , bethanechol;  $n = 7$ , ATP).

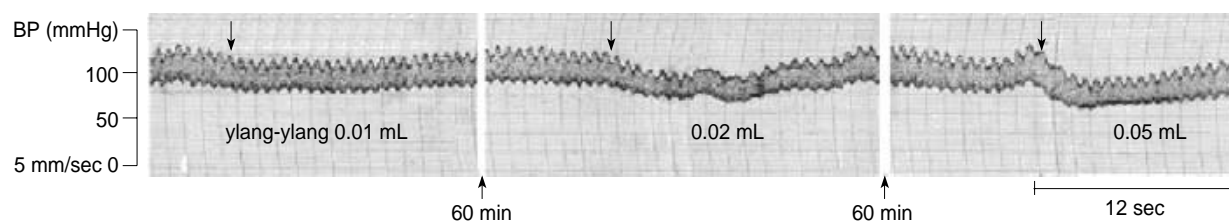


Fig. 5. Representative figure showing the effects of intra-arterial ylang-ylang (0.01-0.05 mL) on blood pressure. Administration of ylang-ylang decreased the amplitude of blood pressure transiently.

of it on detrusor muscle contractibility.

In this study, *in vitro* studies using rat bladder were performed in order to investigate the basic effect of YY oil. Because of rat's a little body volume, a relative large amount of YYEO which was diluted in 0.1% ethanol 1 mL and 0.1% ethanol itself, *in vivo* studies using the bladder of rats resulted in large errors on the change of intravesical pressure and were impossible to check the change of blood pressure during the study. Thus trials by larger animals were needed to obtain error-free results and to observe the adverse effect on the cardiovascular system.

In the results of this study, YYEO inhibited all stimulation by EFS and all drugs to contract the bladder. The results were the same in *in vitro* as well as in *in vivo* studies. When large amounts, up to 0.5 mL of YY oil was administrated intravenously, only transient and mild decrease of blood pressure was found. This suggests that the side effect on the cardiovascular system did not lead to a decrease of intravesical pressure.

Many experiments have been carried out to determine the effect of this oil. Elizabetsky et al. (15) used linalool in white rats to show depression of motor neuron stimulation as well as affecting the central nervous system (CNS) directly. The mechanism of this effect is that the linalool combines with glutamine, CNS excitatory neurotransmitter and inhibits its action. Also in Buchbauer's study (16), essential oils have a selective effect on tissues, but as it has a nonspecific toxic effect on cell membranes, it has no vital activity. However, in different studies, effects on smooth muscle relaxation were investigated. Hume (17, 18) exerts its effect by  $[K^+]_o$  on L-type  $Ca^{2+}$  channels. Eugenol works as  $Ca^{2+}$ -antagonist. Eugenol depressed the constrictor response to norepinephrine (NE), histamine and transmural nerve stimulation. Since NE and histamine act on different and specific receptors, it is probable that dilator effect of eugenol was not mediated by the competition for specific receptor site, but it inhibited one or more stage of the post-receptor excitation-contraction sequence in smooth muscle (19). Recently, it is seen that the eugenoldiol, derived from natural eugenol made a role as a stimulator of non-selective  $\beta$ -adrenoreceptor blocker and  $\beta$ -adrenoreceptor blocker or as a  $\beta$ 2-adrenoreceptor selective partial agonist (17, 20-22). In this experiments, we used crude oil because we had have the purpose that this oil will be used in aromatherapy treatment applied via the skin through massage or add to bath water or by inhalation. There is some degree of absorption of the individual components into the blood, which was calculated at between 4 and 25% when applied to the skin of a rhesus monkey or man (23). This would be approximately  $8 \times 10^{-6}$  and  $1 \times 10^{-5}$  g/mL of an essential oil in carrier oil applied.

The smooth muscle relaxation was made by c-AMP related by activation of adenylate cyclase and c-GMP related to activation of guanylate cyclase in nitric oxide pathway (24). Lis-Balchin and Hart's study (25) showed that smooth mus-

cle relaxation by lavender essential oils was occurred by the elevated c-AMP levels within the cell. In this study, the action mechanism of YYEO in bladder contractility reduction is thought to increasing c-AMP level because the c-AMP non-specific inhibitor, N-ethylmaleimide only inhibited the reduction of bladder contractility by YYEO, but inhibitors related to nitric oxide pathway did not.

In summary, YYEO is shown to have a relaxing effect on bladder smooth muscle. The action mechanism of YYEO is thought through the c-AMP pathway; however the exact mechanism is still unclear. YYEO have several key constituents, one of which would be believed to produce its therapeutic effect. Therefore, to clarify the YYEO in the clinical application and make the products without the loss of its natural scent, further research is needed to determine the effects of each key components of the YYEO.

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